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ORIGINAL ARTICLE

Increased intracellular and extracellular oxidant production in phagocytes of rheumatic patients treated with biological therapy – whole blood quantification

Viera JANČINOVÁ [1], Radomír NOSÁĽ [1], Juraj PAYER [2], Zdenko KILLINGER [2]

[1] 1Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Bratislava, Slovak Republic
[2] 5th Department of Internal Medicine, Medical Faculty of Comenius University, University Hospital, Bratislava, Slovak Republic

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ABSTRACT

Infectious complications, resulting from reduced activity of immune cells, are the most severe and common adverse effects of biological therapy. This study analyzed the effect of biological therapy on blood phagocytes, focusing on the formation of reactive oxygen species (ROS), an important factor in the defence against invading pathogens. Intra- and extracellular ROS production were recorded separately, on the basis of luminol and isoluminol chemiluminescence in patients treated with antibodies against tumor necrosis factor-α or against interleukin-6 receptor. In comparison to healthy donors or to rheumatic patients treated with classical immunosuppressive drugs, biological therapy increased ROS formation in both compartments. This indicates that the anti-microbial activity of blood phagocytes was not reduced by TNFα- or IL-6-neutralizing therapy, at least in terms of ROS.

The method presented does not require blood fractionation, which could modify activity of phagocytes and cause loss of some subpopulations of these cells. The technique is simple, requires microliter volumes of blood and is thus well applicable to clinical studies.

KEY WORDS: blood phagocytes; intracellular and extracellular chemiluminescence; rheumatic patients; biological therapy

Introduction

Biological therapy represents a highly effective treatment of rheumatic diseases. It suppresses the inflammatory response through various mechanisms, such as targeting pro-inflammatory cytokines (particularly tumor necrosis factor TNFα and interleukin IL-6), interference with intracellular signalling pathways of immune cells, depletion of B cells or control T cell activation (Semerano *et al.,* 2016). However, the reduction in the activity of immune cells increases the risk of infection. Infectious complications are the most severe and common adverse effects of biological therapy (Hartmann *et al.,* 2005).

Reactive oxygen species (ROS) produced by blood phagocytes are essential in defence against invading pathogens. For a long time, ROS have been considered harmful mediators of inflammation owing to their highly reactive nature. However, there is an increasing number of findings suggesting that phagocytic ROS are anti-inflammatory and prevent autoimmune reactions. ROS are now considered as cell activity controllers, inflammation-limiting substances and as regulators capable to fine-tune the inflammatory response, depending on when, at what amounts, and where they are produced (Bjorkman *et al.,* 2008; Hultqvist *et al.,* 2009).

A long-standing paradigm has been that ROS formation occurs exclusively in the plasma membrane invaginations – phagosomes. A growing body of evidence points to the possibility that phagocytes are capable to generate oxidants within intracellular organelles also in the absence of phagocytosis. Recent clinical findings indicate that intracellular and extracellular ROS production are regulated differently and that the generation of intracellular ROS is important for limiting inflammatory reactions (Bylund *et al.,* 2010).

In the present study, extra- and intracellular ROS production was determined separately, using whole blood, *i.e.* in phagocytes unaffected by isolation procedure. The

Correspondence address:
PharmDr. Viera Jančinová PhD.
Institute of Experimental Pharmacology and Toxicology
Slovak Academy of Sciences
Dúbravská cesta 9, 841 04 Bratislava, Slovak Republic
TEL.: +421-2-32295728 • E-MAIL: viera.jancinova@savba.sk

method was applied in the analysis of blood obtained from two groups of rheumatic patients – treated with classical or with biological therapy, and blood of healthy donors.

Materials and methods

Chemicals

Luminol, isoluminol and superoxide dismutase (SOD) were from Sigma-Aldrich Chemie (Deisenhofen, Germany). Horseradish peroxidase (HRP) and catalase (CAT) were obtained from Merck (Darmstadt, Germany). All other products are available commercially or their origin is mentioned in the text. Tyrode's solution consisted of 136.9 mmol/l NaCl, 2.7 mmol/l KCl, 11.9 mmol/l $NaHCO_3$, 0.4 mmol/l $NaH_2PO_4.2H_2O$, 1 mmol/l $MgCl_2.6\ H_2O$ and 5.6 mmol/l glucose, pH 7.4.

Blood collection and white blood cell counting

All measurements were made using blood samples from healthy donors (n=7), who had not received any medication for at least seven days, and samples of patients suffering from rheumatoid arthritis (n=23) or ankylosing spondylitis (n=6). According to the therapy, the patients were divided into two groups. The group of biological therapy (bDMARDs) involved 21 patients treated with TNFα-neutralizing antibodies (adalimumab, golimumab, etanercept) or with an antibody against the interleukin-6 receptor (tocilizumab). The group of conventional therapy (csDMARDs) consisted of eight patients medicated with methotrexate, methylprednisolone, sulfasalazine or with the combinations of these drugs. Fresh blood was obtained by venepuncture and anticoagulated with 3.8% trisodium citrate (blood: citrate ratio 9:1). The number of white blood cells was determined by the analyzer ABX Pentra 60 (Horiba Medical, Irvine, CA, USA).

Intracellular and extracellular chemiluminescence

Chemiluminescence was measured in a microtiter plate luminometer LM-01T (Immunotech, Czech Republic) for 60 min at 37 °C. Measurement of intracellular chemiluminescence was performed in samples containing luminol (final concentration FC 100 µmol/l), HRP (FC 8 U/ml), superoxide dismutase (FC 100 U/ml), catalase (FC 2 000 U/ml), Tyrode's solution and blood (FC 1 µl/ml), each component in a 50 µl aliquot. Extracellular chemiluminescence was enhanced with isoluminol (FC 100 µmol/l) and scavengers SOD and CAT were omitted. Peroxidase was added to ensure complete ROS detection. The enzyme is essential for the oxidation of luminophores (Nakamura *et al.*, 1998) and therefore the formation of chemiluminescence signal may be limited by insufficient secretion of peroxidase from neutrophil granules.

Data analysis

Production of ROS by blood phagocytes was evaluated on the basis of integral values of chemiluminescence (area under chemiluminescence curve). All values are given as the means ± SEM. The statistical significance of differences between means was established by Student's t-test.

Results

Effect of blood volume, extracellular peroxidase and extracellular scavengers on luminol and isoluminol chemiluminescence

The intensity of the chemiluminescence signal decreased with increasing volume of blood in the samples. Maximum chemiluminescence was recorded at blood dilution of 500 or 1 000 times, *i.e.* when the 250 µl sample contained 0.5 or 0.25 µl of blood (Figure 1). The data presented in Table 1 show the differences between luminol and isoluminol chemiluminescence. The isoluminol-enhanced chemiluminescence was found to be completely related to extracellular ROS due to its complete blockade by extracellular scavengers and its complete dependence on extracellular peroxidase; in the absence of HRP the isoluminol

Table 1. Effect of extracellular peroxidase (HRP, horseradish peroxidase) and extracellular scavengers (SOD/CAT, superoxide dismutase and catalase) on isoluminol- and luminol-enhanced chemiluminescence of human whole blood.

	Chemiluminescence (RLU)		
	No addition	HRP	HRP + SOD/CAT
Isoluminol[a]	81 105 ± 1 159	343 407 ± 15 307	83 342 ± 1 291
Luminol[a]	118 601 ± 2 466	3 041 054 ± 120 139	132 456 ± 3 035

[a]Background = 78 070 ± 1 079 RLU
Samples contained blood diluted 1 000 times, isoluminol or luminol (100 µmol/l). Superoxide dismutase (100 U/ml), catalase (2 000 U/ml) and/or horseradish peroxidase (8 U/ml) were added as indicated. Mean integral values of chemiluminescence over 60 min are given. RLU = relative light units. Mean ± SEM, n=28.

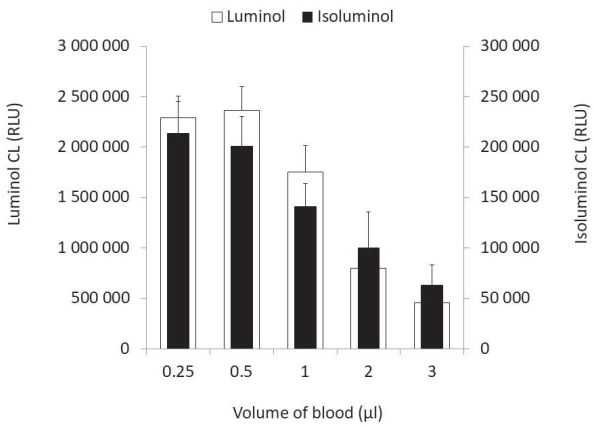

Figure 1. Effect of blood volume on luminol- and isoluminol-enhanced chemiluminescence (CL) of human whole blood. Samples (250 µl) contained from 0.25 to 3 µl of blood, HRP (8 U/ml), luminol or isoluminol (100 µmol/l). Chemiluminescence was recorded continuously for 60 min at 37 °C. Columns represent mean integral values of chemiluminescence (area under chemiluminescence curve). Mean ± SEM, n=4–8, RLU – relative light units.

chemiluminescence was at the background level. In contrast, luminol chemiluminescence was partially resistant to the effect of extracellular scavengers, indicating that a portion of luminol crosses biological membranes and produces chemiluminescence inside phagocytes.

Effect of biological therapy on extra- and intracellular ROS production in blood phagocytes of rheumatic patients

In patients, the spontaneous intracellular ROS formation was more pronounced than in controls (Figure 2A). The chemiluminescence produced inside phagocytes was increased by 164% and by 283% in patients on classical and biological therapies, respectively. Phagocytes of patients treated with biologics (bDMARDs) produced significantly more oxidants into extracellular space (Figure 2B); the extracellular chemiluminescence was elevated by more than 40% in comparison to controls and csDMARDs patient group. There were no significant differences in the total white blood cell number or in neutrophil, lymphocyte, monocyte, eosinophil and basophil counts, on comparing two patient groups and controls (Table 2).

Discussion

The method presented is based on different capacities of luminol and isoluminol to cross biological membranes, resulting from different physico-chemical properties of these luminophores (Dahlgren *et al.,* 1999 and 2007). The molecule of isoluminol is more hydrophilic and more polar than that of luminol, as indicated by values of solvation, dipole moments, lipophilicity parameters, and by the lower capacity of isoluminol to form intramolecular hydrogen bridges (Jančinová *et al.,* 2006). This results in reduced membrane movement an in accumulation of isoluminol in extracellular space. The extracellular origin of isoluminol chemiluminescence was confirmed by its complete blockade in the presence of extracellular scavengers and its complete dependence on extracellular peroxidase – in the absence of HRP the isoluminol chemiluminescence was at the background level (Table 1). In contrast, the molecule of luminol is lipo-hydrophilic in nature and it generated chemiluminescence which was partially resistant to the effect of extracellular scavengers. This indicates

Table 2. Number of white blood cells established in healthy donors and in rheumatic patients treated with classical (csDMARDs) or biological (bDMARDs) therapy.

	Number of white blood cells (10^3/µl)					
	Total	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
Controls	5.77±0.68	3.38±0.45	1.77±0.21	0.44±0.04	0.14±0.03	0.02±0.010
csDMARDs	6.30±0.32	3.85±0.34	1.70±0.20	0.45±0.06	0.26±0.09	0.03±0.003
bDMARDs	6.14±0.31	3.42±0.24	1.95±0.14	0.52±0.03	0.22±0.02	0.02±0.003

The counting was done using the analyzer ABX Pentra 60 (Horiba Medical, Irvine, CA, USA). Mean ± SEM, n=7-21.

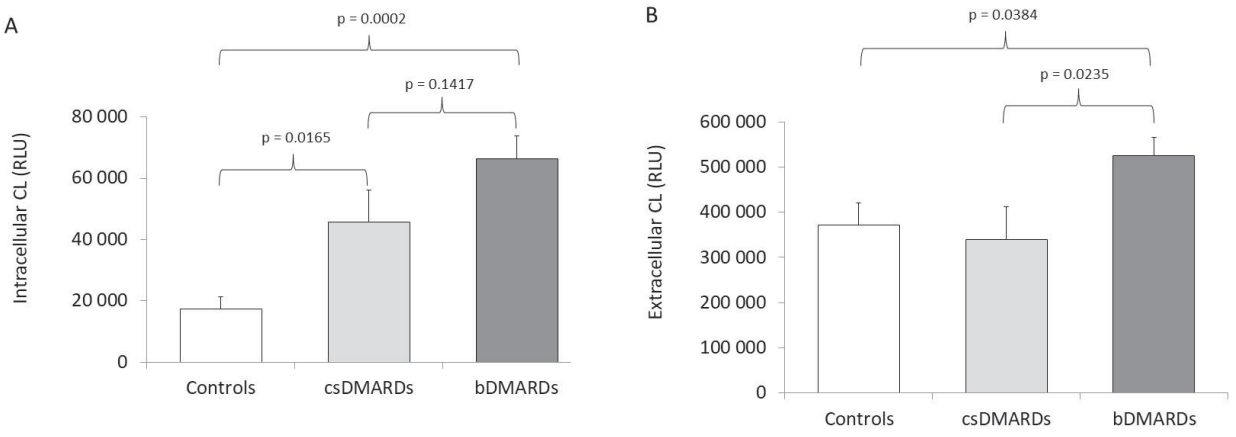

Figure 2. Intracellular (Part A) and extracellular (Part B) chemiluminescence produced by blood phagocytes of rheumatic patients treated with biological (bDMARDs) or classical (csDMARDs) therapy compared to the chemiluminescence of healthy donors. Intracellular chemiluminescence was measured in samples containing luminol (100 µmol/l), HRP (8 U/ml), superoxide dismutase (100 U/ml), catalase (2 000 U/ml), and blood (1 µl/ml). Extracellular chemiluminescence was enhanced with isoluminol (FC 100 µmol/l) and scavengers SOD and CAT were omitted. Columns represent mean integral values of chemiluminescence over 60 min. Mean ± SEM, n=7–21, RLU – relative light units.

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that a portion of luminol crosses biological membranes and produces chemiluminescence inside phagocytes. The intracellular portion can be selectively recorded in the presence of superoxide dismutase and catalase.

Neutrophils, the most abundant population of blood phagocytes, are considered to be the crucial source of whole blood chemiluminescence. The correlation between neutrophil count and chemiluminescence was found to be positive and linear. Compared to neutrophils, monocytes contributed to blood chemiluminescence negligibly, due to their 10 times lower number and their 4 times lower ability to produce chemiluminescence; participation of blood platelets was not detectable (Ristola and Repo, 1989). Due to the complexity of blood, consideration should be given to some blood components (*e.g.* hemoglobin, catalase) which decrease the chemiluminescence signal. According to Rájecký *et al.* (2012), this interference can be eliminated by the application of small blood volumes – up to two microliters per sample. In our experiments, the maximum chemiluminescence was recorded in samples containing 0.25 µl and 0.5 µl of blood; the use of larger volumes led to the reduction of chemiluminescence signal (Figure 1).

The whole blood chemiluminescence method was applied to the differentiation between extra- and intracellular ROS production in rheumatic patients. In comparison to controls and to patients treated conventionally, the biological therapy increased formation of ROS in both compartments (Figure 2). This indicates that the antimicrobial activity of blood phagocytes was not reduced by TNFα or IL-6 neutralizing therapy, at least in terms of ROS formation. Increased chemiluminescence was observed by Capsoni *et al.* (2005) in isolated neutrophils of rheumatic patients receiving adalimumab therapy. In other studies, the biological therapy did not exert any effect (Moreland *et al.,* 2002; Den Broeder *et al.,* 2003; Hartmann *et al.,* 2005). The differences might result from the use of neutrophils that have undergone isolation procedure. Blood fractionating can modify neutrophil activity and cause a loss of some subpopulations of these cells. Alterations in neutrophil subpopulations, known to occur in chronic inflammation (Kolaczkowska and Kubes, 2013; Thieblemont *et al.,* 2016), are not necessarily optimally revealed by testing purified neutrophils.

The presented results, initial and coming from a small group of patients, showed increased ROS formation in blood phagocytes during biological and conventional therapy. This is in line with an increasing number of findings suggesting that phagocytic ROS are anti-inflammatory and prevent autoimmune reactions (Bjorkman *et al.,* 2008; Hultqvist *et al.,* 2009; Bylund *et al.,* 2010). Clinical and experimental observations indicate that absent or compromised phagocytic ROS production results in hyper-inflammation instead of milder inflammatory response, as would be expected in light of the still prevailing opinion. For example, chronic granulomatous disease (*i.e.* inability to assemble a functional NADPH oxidase, CGD) is characterized by absent or subnormal levels of ROS and by recurrent infections associated with hyper-inflammatory and autoimmune manifestations. CGD patients are more susceptible to autoimmune diseases such as rheumatoid arthritis, lupus or Crohn's disease (Bjorkman *et al.,* 2008; Hultqvist *et al.,* 2009; Bylund *et al.,* 2010). Future analysis, based on a more extensive data set, could clarify the relationship between increased ROS production in blood phagocytes and the beneficial anti-inflammatory effect of biological therapy.

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